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Efficient and rapid protein expression and purification of small high disulfide containing sweet protein brazzein in *E. coli*

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Abstract

Brazzein protein comes from an edible fruit, which has a long history of being a staple in the local human diet in Africa. The attractive features of brazzein as a potential commercial sweetener include its small size (53 amino acid residues), its stability over wide ranges of temperature and pH, and the similarity of its sweetness to sucrose. Heterologous production of brazzein is complicated by the fact that the protein contains four disulfide bridges and requires a specific N-terminal sequence. Our previous protocol for producing the protein from *Escherichia coli* involved several steps with low overall yield: expression as a fusion protein, denaturation and renaturation, oxidation of the cysteines, and cleavage by cyanogen bromide at an engineered methionine adjacent to the desired N-terminus. The new protocol described here, which is much faster and leads to a higher yield of native protein, involves the production of brazzein in *E. coli* as a fusion with SUMO. The isolated protein product contains the brazzein domain folded with correct disulfide bonds formed and is then cleaved with a specific SUMO protease to liberate native brazzein. This protocol represents an important advancement that will enable more efficient research into the interaction between brazzein and the receptor as well as investigations to test the potential of brazzein as a commercially viable natural low calorie sweetener.

Keywords: Brazzein; Sweet protein; Low calorie protein sweetener; Disulfide bonds; Csαβ family; Bacterial expression; SUMO fusion; Soluble fusion; *Escherichia coli*

The affluent countries of the world are suffering from an epidemic of obesity, insulin-resistance, and type II diabetes. In our evolutionary past, a strong drive to find rich energy sources and high carbohydrate foods was an advantage for survival. Today, with lives of a more sedentary nature, this sweet-seeking behavior has now become a liability, and low-calorie sweeteners with good taste properties are becoming more sought after, particularly naturally occurring ones, such as proteins.

Over the last 30 years, high potency sweet proteins have been identified in a variety of African and South Asian fruits. The first one discovered was thaumatin (22,206 Da) [1]. It was closely followed by monellin (11,086 Da) [2], mabinlin (12,441 Da) [3], and more recently brazzein (6473 Da) [4]. Although these three sweet proteins exhibit no sequence or structural homology, they appear to require the presence of charged residues on the protein surface over a non-contiguous area [5,6–10]. It is thus likely that protein sweeteners share similar receptor binding interaction sites. Among the natural, low-calorie sweeteners, brazzein is the most promising because of its superior taste quality and its physical properties. Brazzein contains no carbohydrate and bears no structural resemblance to sucrose. Recombinant brazzein is 2000 times sweeter than sucrose solution on a

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weight–weight comparison (17,000 times higher on a per-molecule basis) [11].

Although taste perception and taste preferences have a rich history of study, it is only in the last few years that the receptors thought to underlie this behavior have been identified. Brazzein is perceived as sweet by humans, old world monkeys, and apes, but not new world monkeys or other tested species. This species difference was exploited in the discovery of the human sweet receptor when it was found that substitution of human T1R2 in mice generated animals with humanized sweet taste preferences [12]. Two receptor gene products, T1R2 and T1R3, are required for an animal's preference for sweet tasting molecules [13].

To understand structural and chemical properties responsible for brazzein sweetness, we engineered a synthetic gene to express the brazzein molecule and developed the first production system for brazzein in bacteria based on the nuclease fusion system developed in our laboratory [11]. We used this approach to discover mutants with sweet-taste properties different from those of the wild-type protein [5]. The major drawbacks of the nuclease fusion system are the initial insolubility of the protein product, the requirement for refolding brazzein, and inefficiency in removal of the fusion tag. We report here a new method based on the SUMO[1] fusion system that supports more efficient production of brazzein. The major advantage of the SUMO fusion system is that it yields brazzein in folded and soluble form in high yield. This method of production will allow us to more rapidly produce brazzein with lower cost for both research studies and future large-scale production. By learning more about its mechanism of action and by developing more potent brazzein variants, we will be in a better position to evaluate brazzein as a potential sugar substitute as a means for fighting problems related to obesity and diabetes.

Methods

Construction of SUMO-brazzein expression vector

The linearized pSUMO vector with BsaI and BamHI restriction sites and T7 promotor and kanamycin resistance [14] was purchased from LifeSensors (LifeSensors, Malvern, PA). The codon optimized synthetic gene designed for bacterial expression of brazzein [11] was cloned between the BsaI (5′-GGTCTC) unique cloning site, downstream and in-frame with the SUMO gene, and the BamHI (3′-GGATCC) site in the multiple cloning site of the pSUMO vector. The pSUMO vector is derived from pET24d and contains the T7 promotor induced with IPTG. The fusion gene coded for a His_6-tagged at the N-terminus of the SUMO protein followed by brazzein at the C-terminus. The new pSUMO-brazzein expression vector was transformed into *E. coli* DH5α strain (Invitrogen). The correct gene sequence was confirmed by sequencing at the University of Wisconsin-Madison Biotechnology Center.

Protein expression of SUMO-brazzein

A variety of expression strains were tested: Rosetta (DE3) from Novagen and BL21 (pLysS), BL21-CodonPlus (DE3)RI, BL21-CodonPlus (DE3)RIP, and BL21-CodonPlus (DE3)RIPL from Stratagene. Cells were grown on Luria broth (LB) growth medium [15] supplemented with an antibiotic (34 μg/mL kanamycin). Of the host cells studied, only BL21-CodonPlus (DE3)RIPL produced high amounts of the SUMO-brazzein fusion protein as detected on Tris–Tricine SDS–PAGE 16% (Invitrogen), and this strain was used for high level protein production.

The SUMO-brazzein fusion protein was expressed by induction with 0.5 mM IPTG at about mid-log phase ($OD_{600nm} \sim 0.6$). The optimum induction time was 24 h, and the temperature was shifted to 25 °C after IPTG addition. Cells from the rich media were harvested at a final OD_{600nm} of 5–6, and yielded 5–6 g of wet cell paste per liter culture. Cells were frozen and kept at −80 °C for later purification procedures.

To verify protein expression, we used SDS–PAGE (Novagen) gel electrophoresis: 1.0-ml aliquots were removed before IPTG induction (at $OD_{600nm} \sim 0.6$) and at 24 h post-induction (the latter aliquot was diluted 1:6 or 1:8 to adjust the cell concentration to that of the earlier sample). Cells were centrifuged at 6000 rpm for 2 min, and the isolated pellet was resuspended by adding 50 μl distilled water and 50 μl 2× Tricine sample buffer (contains 200 mM Tris–HCl, 2% SDS, 40% glycerol, and 0.04% Coomassie Brilliant Blue G-250 (pH 6.8) and β-mercaptoethanol). Cell disruption, denaturation, and disulfide reduction were allowed to proceed for 5 min before the sample was loaded onto the gel. The running buffer was Tris–Tricine; gel staining was with R250 Coomassie blue.

Purification of the His_6-SUMO-brazzein fusion

We used Ni–NTA superflow resin (*Qiagen*, Valencia, CA) under native conditions to quickly purify the soluble His_6-SUMO-brazzein fusion from the cell lysate. Cells were first suspended by adding either BugBuster (Novagen) according to the manual or lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 6.8) complemented with 300 μg/mL lysozyme and 90 μg/mL PMSF. The suspensions were kept on ice to facilitate cell lysis. For complete lysis, cells were sonicated on ice by 2 periods each of 4 min (cycles of 10 s on followed by 30 s off). Cells were centrifuged at 10,000*g* for 20 min at 4 °C. The supernatant was applied to the pre-equilibrated Ni–NTA column

[1] *Abbreviations used:* IPTG, isopropyl-β-D-thiogalactopyranoside; Ni-NTA, nickel–nitrilotriacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; RP-HPLC, reversed phase high pressure liquid chromatography; TFA, trifluoroacetic acid; SUMO, small ubiquitin-like modifier; DTT, dithiothreitol; 2D-NOESY, two-dimensional nuclear Overhauser spectroscopy; NMR, nuclear magnetic resonance.